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PHAGOCYtic LINING CELLS DETERMINE LOCAL EXPRESSION OF INFLAMMATION IN TYPE II COLLAGEN-INDUCED ARTHRITIS

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Objective. To investigate the *in vivo* role of phagocytic synovial lining cells in the local expression of inflammation in type II collagen-induced arthritis (CIA) in DBA/1J mice.

Methods. On various days before arthritis induction (day 7, 5, or 2), phagocytic lining cells were selectively depleted from the synovial layer by injecting multilamellar liposomes containing clodronate (dichloromethylene diphosphonate) directly into the knee joints. As controls, either PBS or PBS-laden liposomes were injected. CIA was induced by immunizing mice with heterologous bovine type II collagen in Freund's complete adjuvant. Arthritis onset was synchronized by a single intraperitoneal injection of lipopolysaccharide; arthritis was evaluated in hematoxylin and eosin-stained knee joint sections. Chemotactic activity in synovial washout samples was detected in a Transwell chemotactic assay. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) protein levels were measured in NOB-1 and L929 bioassays, respectively. IL-1 messenger RNA (mRNA) in synovial specimens was measured by reverse transcriptase-polymerase chain reaction. IL-1 was also detected immunohistologically in knee joint sections.

Results. In clodronate-laden liposome-treated, lining-depleted knee joints, there was significantly decreased inflammation compared with controls. Cell influx into the synovium was markedly decreased. Expression of IL-1 mRNA in the synovium was significantly

reduced. IL-1 was detected only in some cells in the deeper synovial layer, in contrast to controls, in which large numbers of cells in the deeper synovial layer were stained. In synovial washouts from lining-depleted knee joints, biologically active IL-1 levels were reduced by 40% at 6 hours after arthritis induction. Most strikingly, chemotactic activity was highly decreased in these synovial washout samples. When IL-1 or TNF α was injected into the knee joints of immunized mice in which arthritis was not yet expressed, arthritis was not induced in the lining-depleted joints, whereas marked cell influx was found in control joints.

Conclusion. Our data indicate that phagocytic lining cells play a crucial role in the local expression of inflammation in systemically induced CIA. Phagocytic lining cells probably form an important source of chemotactic factors which are set free upon activation by IL-1 or TNF α .

Type II collagen-induced arthritis (CIA) is a generally accepted animal model of rheumatoid arthritis. This type of arthritis can be evoked in mice or rats with homologous or heterologous type II collagen and is thought to be mediated by immune complexes (1,2) and T cells (3). Early CIA is characterized by the influx of mainly macrophages into the synovial layer, degradation of cartilage matrix proteoglycans, and chondrocyte death (4). Cytokines, such as interleukin-1 (IL-1) (5–7) and tumor necrosis factor (TNF) (8,9), are thought to be crucial proinflammatory agents, accelerating arthritis onset and mediating cartilage damage.

The synovium, which covers the inside of diarthrodial joints, forms the main focus of the inflammatory response. In normal joints, the synovium is composed of 2 layers. The sublining layer contains blood vessels and nerves embedded in loose connective tissue. This layer is covered by the thin lining layer, which contains the fibroblast-like type B and the macrophage-like type A

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cells, the latter having a strong phagocytic capacity (10,11).

Phagocytic lining cells can be depleted from the lining layer prior to arthritis induction in order to investigate their *in vivo* role in the onset of arthritis. Recently, a technique was developed to selectively deplete macrophages from tissues by the use of liposomes containing the drug clodronate (12). We applied this technique to the murine knee joint, where local treatment eliminated the phagocytic lining cells covering the sublining layer (13). Optimal depletion was found between days 5 and 7 after clodronate-laden liposome injection; the cells slowly returned thereafter (13).

The aim of the present study was to examine the effect of lining depletion on the onset of CIA in mice. Since the onset of arthritis within the knee joints of murine CIA is unpredictable and may vary between days 28 and 50 after immunization, arthritis onset was synchronized by giving a single injection of lipopolysaccharide (LPS) on day 28, as described elsewhere (14). Lining depletion was done either on day 7, 5, or 2 before LPS challenge. We investigated the arthritis histologically. Chemotactic activity, IL-1 levels, and TNF α levels were measured in synovial washout samples; IL-1 was localized immunohistologically.

Our results indicate that depletion of phagocytic lining cells significantly blocks the influx of inflammatory cells. IL-1 messenger RNA (mRNA) and protein levels were significantly decreased. Injection of IL-1 or TNF into lining-depleted knee joints failed to induce cell influx compared with controls. Because the chemotactic activity in synovial washouts of lining-depleted arthritic joints was significantly decreased, this may indicate that such a joint loses its capacity to produce chemotactic factors.

MATERIAL AND METHODS

Animals. Male DBA/11acJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed under semisterile conditions, were fed a standard diet and tap water *ad libitum*, and were used between 10 and 12 weeks of age.

Induction of arthritis. Mice were immunized with bovine type II collagen (100 μ g) emulsified in Freund's complete adjuvant (*Mycobacterium tuberculosis* 2 mg/ml; Difco, Detroit, MI), by a subcutaneous injection into the base of the tail. The animals were given a booster injection on day 21 with 100 μ g of type II collagen intraperitoneally. The onset of polyarthritis occurs around 4–5 weeks; however, the onset, incidence, and severity of the arthritis is highly variable within and among experiments.

To synchronize the onset of arthritis in the knee joint, 40 μ g of bacterial LPS was given intraperitoneally on day 28

after immunization. LPS greatly potentiates the standard CIA model, possibly by inducing the release of endogenous IL-1 (14); 48 hours after LPS administration, inflammation of the paws is observed macroscopically. To investigate the effect of local activation by cytokines, 100 or 200 ng of recombinant IL-1 β or TNF α , instead of LPS, was injected into the knee joint on day 28 after immunization.

Liposome-encapsulated dichloromethylene diphosphate (Cl₂MDP). Multilamellar liposomes encapsulating the drug clodronate, or Cl₂MDP, were prepared as described previously (15). Clodronate was kindly provided by Boehringer (Mannheim, Germany). Briefly, 86 mg of phosphatidylcholine and 8 mg of cholesterol were dissolved in chloroform in a round-bottom flask. By vacuum rotary evaporation at 37°C, a thin film formed on the wall of the flask. Clodronate (2.5 gm) was dissolved in 10 ml of PBS, with gentle shaking for 10 minutes, kept at room temperature for 2 hours, and was then sonicated at 20°C for 3 minutes. After an additional 2 hours at room temperature, free clodronate was removed by washing 3 times with PBS (20,000g for 10 minutes). The natural fate of liposomes is phagocytosis by phagocytic cells.

Depletion of phagocytic lining cells with clodronate-laden liposomes. Six microliters of a liposome suspension containing 30 μ g of Cl₂MDP was injected into the knee joints of type II collagen-immunized mice on day 7, 5, or 2 before arthritis onset (*n* = 20 per group). As controls, PBS or PBS-containing liposomes were injected (*n* = 20 per group). Phagocytic synovial lining cells ingest the clodronate-laden liposomes, the membrane of the liposome is disrupted during digestion, the clodronate is set free, and it kills the cell. Injections of clodronate-laden liposomes into the knee joints of control C57Bl/6 mice produced optimal depletion of synovial lining cells by day 7 after injection (13).

Treatment protocol and macroscopic scoring of arthritis. From day 2 until day 40 after the start of arthritis, clinical severity of arthritis was graded on a scale of 0 to 2 for each hindpaw, according to changes in redness and swelling: 0 = no change, 0.5 = slight, 1.0 = moderate, 1.5 = marked, and 2.0 = maximal swelling and redness. Six and 12 days after the onset of arthritis, mice were killed, and the knee joints were isolated and processed for histology.

Histology. At the end of the experiments, total knee joints were dissected, fixed in 4% formaldehyde, and processed for histology as described previously (16). Standardized frontal sections of total knee joints (7 μ m) were cut and stained with hematoxylin and eosin. Synovial infiltrates and exudate cells were scored on 5 semiserial sections of each specimen, spaced 140 μ m apart. In each section, infiltrate and exudate were graded left and right in the area ranging from the patella to the tibia. Scoring was done in a blinded manner by 2 observers (PLEMvL and AEMH); the numbers provided by the 2 observers represent the mean plus the standard deviation. Synovial infiltrates and joint cavity exudates were graded on a scale of 0–3, according to the amount of inflammatory cells in synovium and cavity, respectively: 0 = no influx, 1 = minor influx, 2 = marked influx, and 3 = maximal influx (the most that was observed in a given set of experiments).

Immunolocalization. *Immunostaining of polymorphonuclear cells (PMNs).* Total knee joint sections were stained with NIMP-R14, a specific rat anti-mouse PMN monoclonal

antibody (a kind gift from Dr. M. Strath, National Institute for Medical Research, London, UK). Briefly, paraffin sections were deparaffinized in xylene, treated with PBS for 10 minutes, and treated with 0.1% trypsin in 0.1% CaCl_2 (pH 7.8). Sections were washed twice in PBS and preincubated with 20% normal rabbit serum in PBS for 15 minutes. In addition, they were incubated with NIMP-R14 (dilution 1:10) in 1% BSA-PBS for 60 minutes, washed twice in PBS, and incubated with rabbit anti-rat peroxidase (1:50) in 5% normal mouse serum (NMS) IgG-PBS for 30 minutes. After washing in PBS, the substrate 3-amino-9-ethylcarbazole, 40 mg/ml in N,N-dimethylformamide (Sigma, St. Louis, MO), was added. Ten minutes thereafter, sections were again washed and counterstained with hematoxylin and eosin and embedded in 8% glycerol-gelatin. Brown-stained cells were counted using an ocular with a 10×10 mm grading system.

Immunostaining for IL-1. IL-1 was detected as described previously (17). Briefly, deparaffinized sections were treated with 1% H_2O_2 in PBS, rinsed in water, and incubated overnight with the primary antibody, rat anti-IL-1 β antiserum, or irrelevant rat antibody at 4°C in a humidified box. Additionally, they were washed and incubated with biotinylated secondary antibody, rabbit anti-rat antibody diluted 1:200 in PBS plus 5% nonfat dry milk, and 2% normal mouse serum for 1 hour at room temperature. After another washing, slides were incubated with the ABC reagent (Vectastain ABC kit; Vector, Burlingame, CA) for 30 minutes at room temperature. Slides were then developed with 0.5 mg/ml diaminobenzidine in TRIS HCl, pH 7.6, and 0.02% H_2O_2 for 10 minutes and counterstained with hematoxylin and eosin. Anti-IL-1 antiserum was preincubated on IL-1 β -coated plates 8 times for 20 minutes each at room temperature; normal rat serum was used as a negative control. For control of IL-1 β specificity, antiserum was preincubated on IL-1 α - or BSA-coated plates.

Detection of IL-1 mRNA levels in synovial tissue. *RNA isolation.* Mice were killed by cervical dislocation, and the patella and adjacent synovium were immediately dissected. Two synovium biopsies (1 from the lateral and 1 from the medial side) were taken with a disposable biopsy punch (3 mm diameter; Stiefel, Wachttersbach, Germany). The synovium samples were immediately frozen in liquid nitrogen. Five separate synovium samples were ground into powder using a microdismembrator II (B. Braun, Melsungen, Germany). Total RNA was extracted in 1 ml of TRIzol reagent, an improved single-step RNA method based on the method described by Chomczynski and Sacchi (18).

Polymerase chain reaction (PCR) amplification. One microgram of synovial RNA and the total amount of cartilage RNA was used for reverse transcriptase (RT)-PCR. Messenger RNA was reverse transcribed to complementary DNA (cDNA) using oligo-dT primers and one-twentieth of the cDNA was used in 1 PCR amplification. PCR was performed at a final concentration of 200 μM dNTPs, 0.1 μM of each primer, and 1 unit of *Taq* polymerase (Life Technologies, Gaithersburg, MD) in standard PCR buffer. The mixture was overlaid with mineral oil and amplified in a thermocycler (Omnigene; Hybaid, Teddington, Middlesex, UK). Message for GAPDH and IL-1 β was amplified using the primers described elsewhere (19,20). Samples (5 μl) were taken from the reaction tubes after a certain number of cycles. PCR products were separated on 1.6% agarose and stained with ethidium bromide.

Table 1. Effects of lining depletion on the onset of type II collagen-induced arthritis in DBA/1 mice*

Days before arthritis onset, injection	Exudate, mean \pm SD cells	Infiltrate, mean \pm SD cells
Day 7		
PBS	0.6 \pm 0.4	1.9 \pm 1.1
PBS-liposomes	0.8 \pm 0.3	2.4 \pm 0.8
Cl ₂ -MDP-liposomes	0.5 \pm 0.4	0.8 \pm 0.7†
Day 5		
PBS	1.2 \pm 0.4	2.8 \pm 0.7
PBS-liposomes	0.8 \pm 0.4	2.4 \pm 0.2
Cl ₂ -MDP-liposomes	0.7 \pm 0.4	0.5 \pm 0.5†
Day 2		
PBS	0.7 \pm 0.4	2.3 \pm 0.8
PBS-liposomes	0.7 \pm 0.5	2.1 \pm 1.0
Cl ₂ -MDP-liposomes	0.4 \pm 0.3	0.7 \pm 0.6†

* Cell influx into the joint cavity and synovium in knee joint sections stained with hematoxylin and eosin was scored on a 0–3 scale (0 = no influx, 1 = minor, 2 = moderate, 3 = marked). Clodronate (Cl₂-MDP)-laden liposomes, PBS-laden liposomes, or PBS alone was injected into the knee joint before arthritis induction. Note the significant reduction in cell influx in the synovial layer in these lining-depleted knee joints. Values represent 2 experiments with 10 mice per group.

† $P < 0.05$ versus PBS alone, by Mann-Whitney U test.

Production of inflammatory mediators by synovial tissue. Synovial tissue was isolated in a standard manner by dissection of the patellar tendon and the patellar plate containing the patella, tendons, and synovium. Synovial specimens were isolated 6 and 48 hours after LPS acceleration to measure early cytokine and chemotactic factor production. Mediators were eluted from synovial specimens derived from 6 knee joints and were washed in 2 ml of RPMI for 1 hour at room temperature. Undiluted washout samples were tested for their chemotactic activity for PMNs in the Transwell chemotactic assay (Costar, Badhoevedorp, The Netherlands). Washout samples were tested for levels of IL-1 and TNF α in an IL-1-sensitive (NOB-1 assay) or TNF α -sensitive (L929 assay) bioassay, respectively.

Determination of chemotactic activity. *Isolation of PMNs.* Murine neutrophil granulocytes (PMN) were isolated using gelatin sponges (Willospoon; Will-Pharma, Zwanenburg, The Netherlands), a method developed by Middleton and Campbell (21). After collecting the cells, analysis of the cytospin products showed that the cell suspension predominantly consisted of neutrophil granulocytes (99%).

Chemotactic assay. PMN chemotaxis was measured using 12-well 6.5-mm Costar Transwell culture plate inserts equipped with polycarbonate filters (no. 3415, pore size 3 μm , polyvinyl pyrrolidone free; Costar, Cambridge, MA). Five hundred microliters of culture supernatant was added to the bottom chamber of the Transwell. As a positive control, we used 5% fresh NMS activated with zymosan for 1 hour. As negative controls, we used decompartmented zymosan-activated serum, PBS, and serum proteins whose protein content was similar to the amount of protein present in the culture media. Subsequently, 100 μl of PMN suspension (10^5 cells/ml) was loaded above the filter and incubated for 2 hours

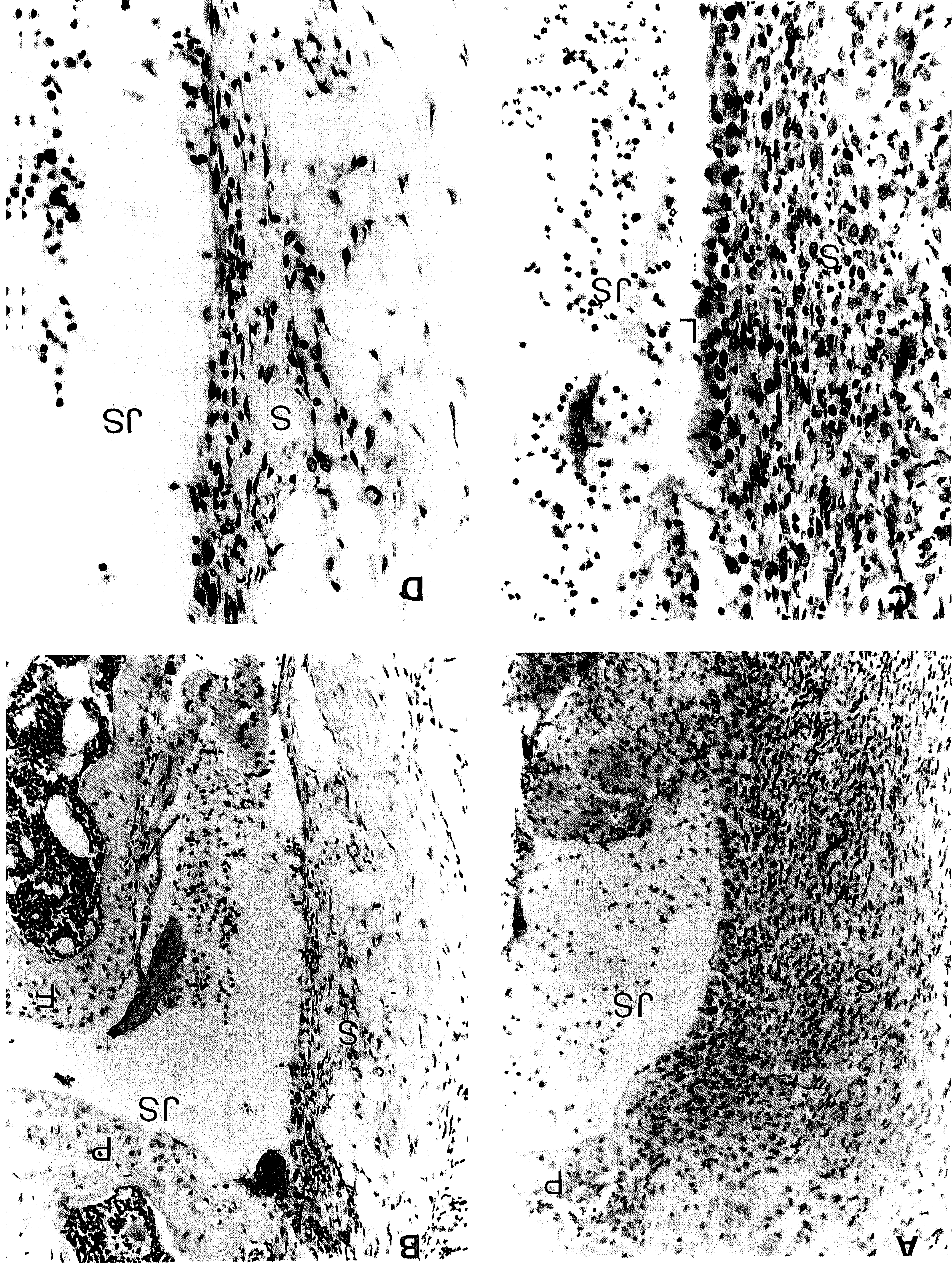


Figure 1. Total knee joint sections from DBA/1 mice immunized with type II collagen, 6 days after arthritis onset. Knee joints were injected intrarticularly with either PBS or PBS-laden liposomes (A and C) or clodronate-liposomes (B and D), 7 days before the onset of arthritis. Note the significantly lower influx of inflammatory cells in the synovium of the lining-depleted arthritic knee joint. S = synovium; P = patella; JS = joint space; F = femur; L = lining. Hematoxylin and eosin stained; original magnification $\times 100$ in A and B; $\times 250$ in C and D.

at 37°C in humidified 5% CO₂. Additionally, cells present in the bottom chamber were counted using a hemocytometer. Results are expressed as the ratio (%) of the number of cells present in the bottom chamber to the number of cells added to the plate insert.

Bioassay for IL-1. IL-1 activity was measured in the 1-stage bioassay for IL-1 as described by Gearing et al (22). The assay was performed as a coculture of the IL-1-specific subclone of the murine thymoma cell EL-4, designated NOB-1, producing IL-2 and IL-4, which activates the lymphokine responder CTLL line. Briefly, NOB-1 cells were washed twice and resuspended at 1×10^6 cells/ml of RPMI containing 5% fetal calf serum. The cells were distributed into 96-well microtiter plates at 2×10^5 cells/well in 100- μ l volumes. Four times 10^3 CTLL cells in 50 μ l RPMI were added, followed by appropriate dilutions of test sample to a final volume of 200 μ l. After 20 hours, 0.5 μ Ci of ³H-thymidine (specific activity 20 Ci/mmol; Amersham, Amersham, UK) was added to each well, and 3 hours later, the contents were harvested and the incorporated activity was determined.

The EL-4 6.1 line, from which NOB-1 was derived, does not incorporate thymidine because it is deficient in thymidine kinase; therefore, only CTLL proliferation is measured by ³H-thymidine incorporation. Maximal ³H-thymidine incorporation in the bioassay in the presence of IL-1 was between 15,000 and 20,000 counts per minute. CTLL alone served as the control and incorporated only 100–1,000 cpm, indicating that the culture media contained only minor concentrations of IL-2 or IL-4.

Bioassay for TNF α . TNF α activity was measured as previously described (23). Briefly, 1×10^5 L929 cells were placed in a flat-bottom 96-well microtiter plate. A standard curve was made by adding serial 2-fold dilutions of recombinant mouse TNF α (range 0.6–0.0002 ng/ml). Arthritic synovial washout samples (dilution 0–3) were tested. After 30 hours of incubation at 37°C in an atmosphere of 5% CO₂, TNF α -mediated cytolytic effects on L929 cells were evaluated. The solution above the cells was gently decanted, and the remaining cells were fixed by adding methanol (96%) for 1 minute. After drying the cells, crystal violet (Sigma) was added, and after 5 minutes, excess crystal violet was washed away. The plate was then dried, 100 μ l of acetic acid was added, the plate was shaken, and the extinction was determined on an enzyme-linked immunosorbent assay reader at 540 nm.

RESULTS

Local expression of inflammation determined by selective depletion of phagocytic lining cells in mouse knee joints. In LPS-accelerated CIA, the incidence of arthritis in both knee joints is >95%. Before LPS acceleration, clodronate-laden liposomes were injected into 1 knee joint and either PBS or PBS-laden liposomes were injected into the contralateral knee joint.

Six days after arthritis onset, knee joints were isolated to study the local expression of inflammation. Hematoxylin and eosin-stained knee joint sections revealed severe synovial inflammation in both PBS- and PBS-liposome-injected knee joints (Table 1). Large

numbers of PMN and monocytes infiltrated into the synovial layer (Figure 1). Infiltration into the joint cavity (exudate) was less prominent. Lining-depleted knee joints showed significantly less synovial cell infiltration, accounting for only 20–30% as compared with controls. The remaining cell influx was mainly found in the superficial layers of the synovium.

The number of monocytes and neutrophils in the synovial layer was determined per high power field (400 \times magnification), using paraffin sections stained with anti-PMN antibodies (NIMP-R14) or hematoxylin and eosin. There were a mean \pm SD of 87 ± 15 monocytes and 49 ± 9 neutrophils in the control sections, and 22 ± 10 monocytes and 13 ± 4 neutrophils in the lining-depleted joints. The ratio of monocytes to PMN was similar in control (1.7) and lining-depleted joints (1.7).

Comparable blockade of cell influx was detected whether lining depletion had started at 7, 5, or 2 days before LPS treatment (Table 1). Local blockade of cell influx within a particular liposome-treated knee joint had no effect on remote expression in the same hindpaw or the paw of the other leg, as illustrated by scores for macroscopic swelling of the hindpaws (Figure 2). To examine whether lining depletion only retarded full-blown CIA expression or had a prolonged suppressive effect, cell influx on day 12 after arthritis onset was determined. Again, a significantly reduced number of inflammatory cells was found, comparable to that seen on day 6 (Table 2). This finding indicates that lining depletion induces a prolonged blocking effect on cell influx within this model.

Since lining depletion per se may provoke arthritis in the presence of an immune background, due to release of cytokines from destroyed cells, we also studied the effect of lining depletion in a group of animals that received no LPS (28 mice). All animals were given clodronate-laden liposomes in 1 knee joint and either PBS-laden liposomes or PBS alone in the contralateral knee joint. Intraarticular injections were given on day 7 before the expected arthritis onset (day 28).

In the nontreated control knee joints, only 14% arthritis incidence was found by histologic examination. Moreover, the severity of inflammation in the knee joint was low. In those mice which did develop arthritis in the control knee joint, cell influx was fully prevented in the contralateral, lining-depleted knee joint. In none of the knee joints in which clodronate-laden liposomes were injected could any signs of inflammation be detected (data not shown). This further indicates that clodronate-laden liposome treatment does not provoke the onset of arthritis.

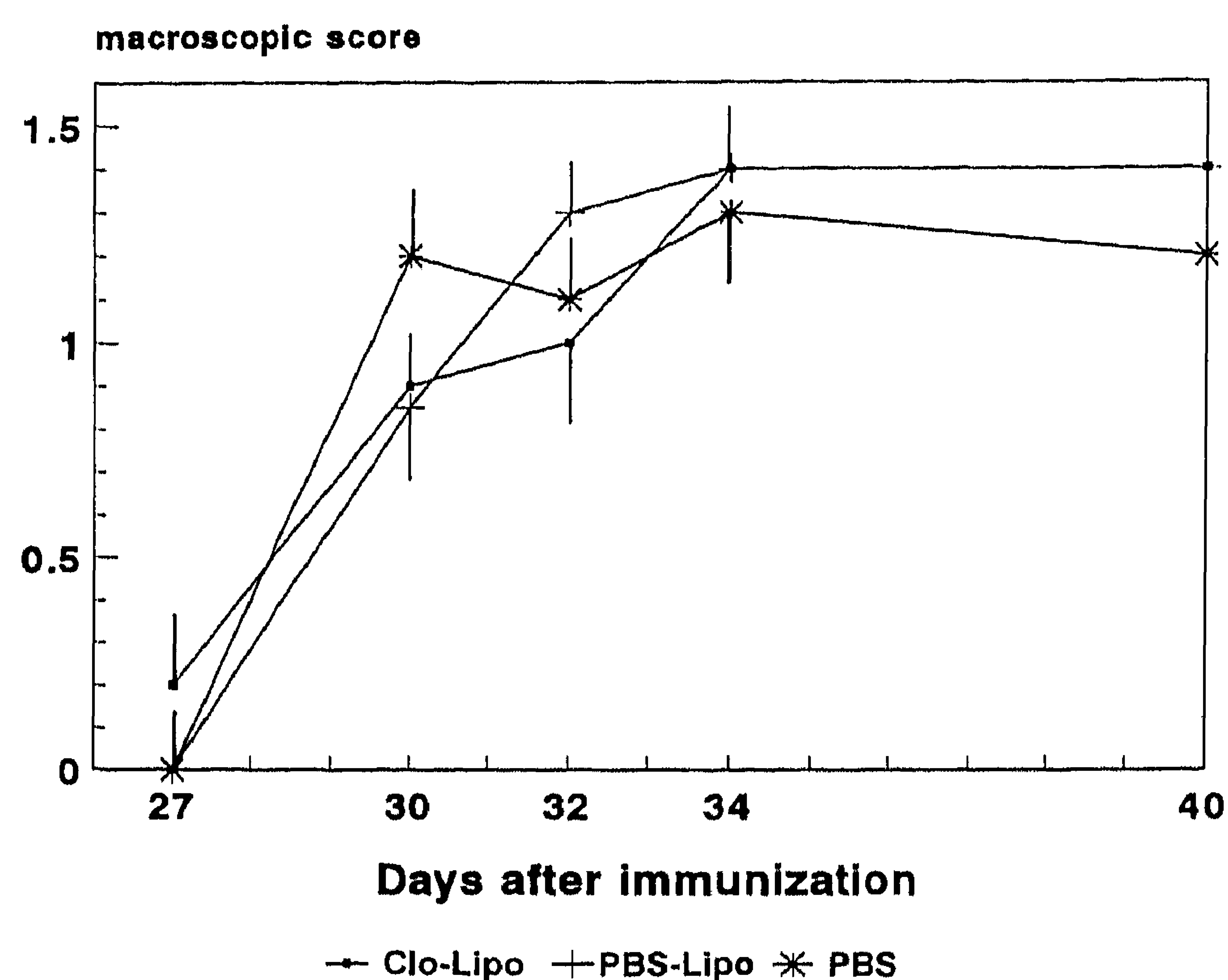


Figure 2. Clinical severity of arthritis in the hindpaws of DBA/1 mice with accelerated type II collagen-induced arthritis (CIA), at several time points after immunization. CIA was accelerated by giving 40 μ g of lipopolysaccharide on day 28. The onset and severity of spontaneous arthritis were highly variable between days 28 and 40 (data not shown). Injection of clodronate-laden liposomes (Clo-Lipo) into the knee joint had no effect on the expression of clinical arthritis in the hindpaw of the same leg. The clinical severity of arthritis in the hindpaws was scored using an arbitrary scoring system (maximum 2). Values are the mean and SD of 14 mice.

Mechanisms involved in decreased influx of inflammatory cells. *Chemotactic activity in synovial washout samples.* Phagocytic lining cells may be important producers of chemotactic factors that are released after stimulation by cytokines such as IL-1 β and TNF α .

Table 2. Effects of lining depletion on the severity of type II collagen-induced arthritis in DBA/1 mice, at early and later time points after arthritis onset*

Days after arthritis induction, injection	Joint cavity exudate, mean \pm SD cells	Synovial infiltrate, mean \pm SD cells
Day 6		
PBS	0.4 \pm 0.2	2.8 \pm 0.8
PBS-liposomes	0.5 \pm 0.4	2.9 \pm 0.5
Cl ₂ -MDP-liposomes	0.2 \pm 0.2	0.5 \pm 0.3†
Day 12		
PBS	0	2.1 \pm 0.4
PBS-liposomes	0.2 \pm 0.4	2.6 \pm 0.8
Cl ₂ -MDP-liposomes	0	0.7 \pm 0.8†

* See Table 1 for details. Note the significant reduction in cell influx in the synovial layer in these lining-depleted knee joints at both times. Values represent 2 experiments with 10 mice per group.

† $P < 0.05$ versus PBS alone, by Mann-Whitney U test.

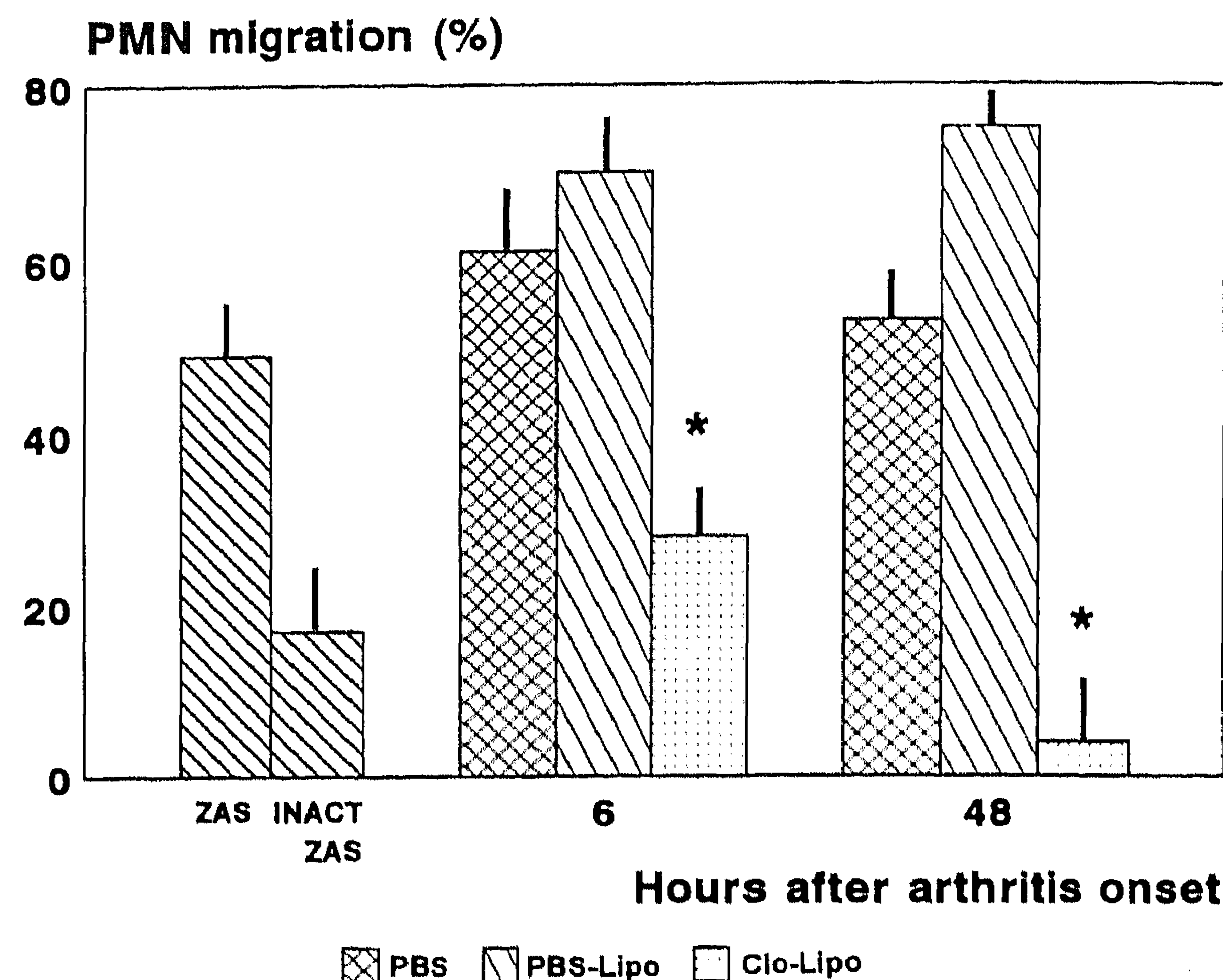


Figure 3. Migration of purified mouse polymorphonuclear cells (PMN) into washouts from the synovium 6 and 48 hours after arthritis induction. Seven days before arthritis induction, knee joints were injected with either PBS, PBS-laden liposomes (PBS-Lipo), or clodronate-laden liposomes (Clo-Lipo; which induced lining depletion). In the Transwell chemotaxis assay, 5% zymosan-activated serum (ZAS) was used as a positive control; decomplexed ZAS (INACT ZAS) was used as a negative control. PBS alone or PBS containing serum protein levels similar to those found in arthritic synovial washouts showed 0–3% PMN migration (data not shown). Values are the mean and SD of 12 mice. * = $P < 0.05$, by Mann-Whitney U test.

Whether elimination of phagocytic lining cells decreases production of chemotactic factors during arthritis onset was studied *ex vivo*. Synovial washout samples from control and lining-depleted knee joints (6 hours and 2 days after arthritis onset) were tested for their ability to attract PMN in a chemotactic assay. Within 1 hour, 55–75% of the PMNs migrated through the filter from the upper to the lower compartment, which contained washouts of control arthritic synovial specimens (Figure 3). Migration of PMN to the synovial washouts derived from lining-depleted arthritic knee joints was significantly lower (30% and 5% of control values at 6 and 48 hours after arthritis onset, respectively).

IL-1 mRNA and TNF/IL-1 protein levels in lining-depleted arthritic knee joints. The decrease of chemotactic activity may be a result of diminished IL-1 β and/or TNF α production. IL-1 has been shown to be a pivotal cytokine, directing the expression of inflammation in CIA (6,7). IL-1 mRNA levels were measured in synovial tissue at 6 hours after arthritis onset, using RT-PCR. In synovia from lining-depleted knee joints, IL-1 mRNA levels were decreased 4-fold compared with controls (Figure 4).

In addition, protein levels of biologically active IL-1 and TNF α were measured in synovial washout specimens derived from lining and control arthritic knee joints. Figure 5 shows that in control joints, significant levels of biologically active IL-1 (~20–40 pg/patella) were detected at 6 hours but not at day 2 after onset of CIA. The latter is related to inhibitory activity. Significantly lower IL-1 levels (up to 40% reduction) were found in lining-depleted arthritic joints. Biologically active TNF α levels were not detected at these time points, indicating that the levels were at or below the limits of detection (20 pg/ml; data not shown).

Using immunolocalization, we examined tissues for IL-1-positive cells 6 and 24 hours after arthritis onset. In control arthritic joints, IL-1 was mainly seen in cells in the sublining area (Figure 6). Only minimal staining was found in the lining layer. Cells stained strongly at 6 hrs but less at 24 hrs after arthritis onset. In lining-depleted knee joints, only few IL-1 positive cells were present in the deeper synovial layers.

Activation of lining-depleted knee joints by intra-articular injection of IL-1 β or TNF α . Whether reduced local levels of IL-1 are directly responsible for decreased cell influx or whether lining cells form an important source of chemotactic factors was further studied. IL-1 β or TNF α (100 ng or 200 ng) was injected into normal as well as lining-depleted knee joints of type II collagen-immunized mice on day 28. No LPS was given. Only those knee joints which showed no clinical signs of spontaneous arthritis at that time point were injected. Two days after cytokine injection, influx of cells in the synovial layer was scored. A severe synovitis was observed after injection of both IL-1 β and TNF α if injected into the control knees of immunized mice (Table 3 and

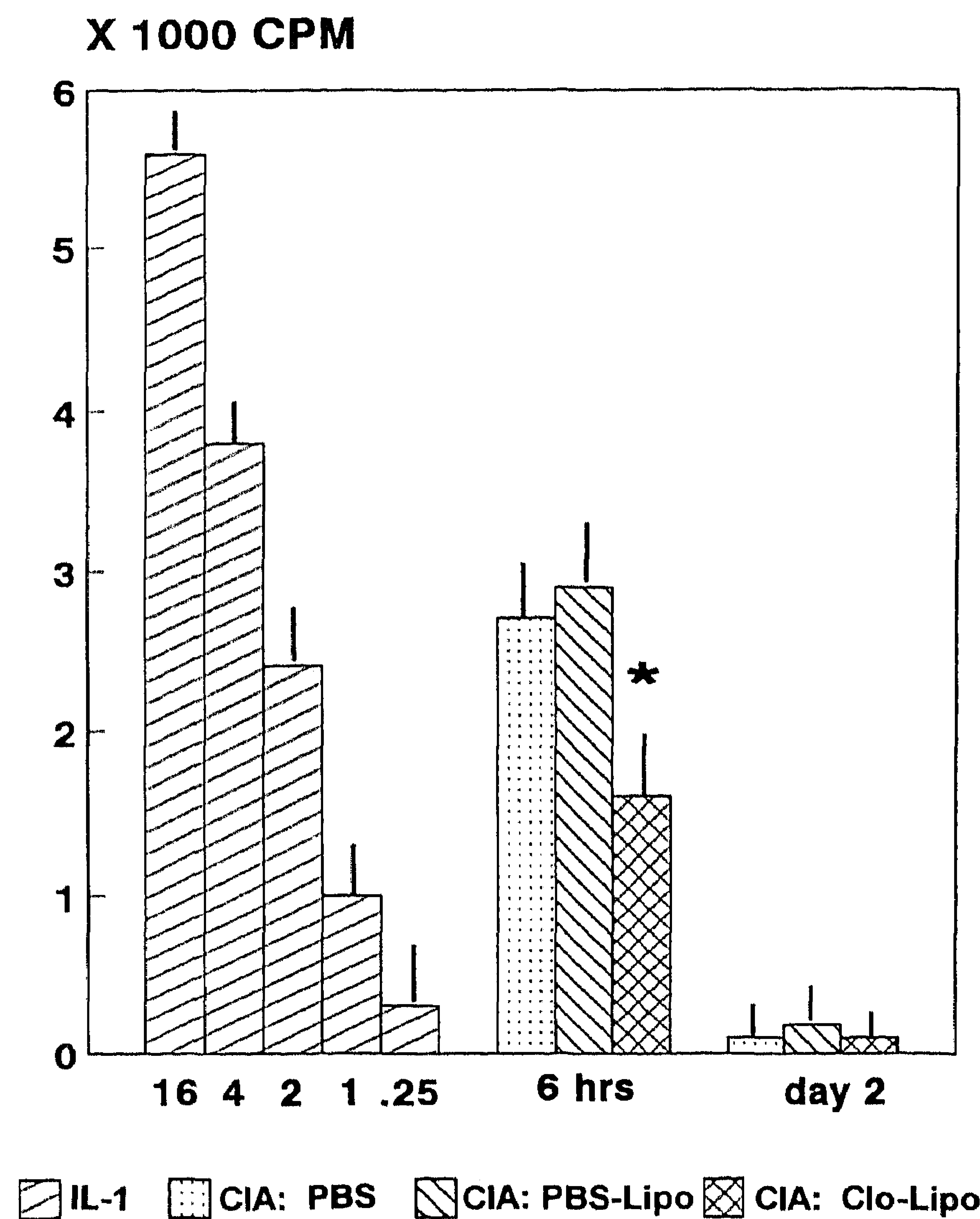


Figure 5. Interleukin-1 (IL-1) production by synovial specimens 6 hours and 2 days after the onset of type II collagen-induced arthritis (CIA) in control and synovial lining cell-depleted knee joints, assayed with NOB-1 cells. Lining cells were depleted by injecting clodronate-laden liposomes (Clo-Lipo) on day 7 before arthritis induction. PBS or PBS-laden liposomes (PBS-Lipo) was injected as a control. As a positive reference control in the bioassay, a dosage range of murine recombinant IL-1 (16, 4, 2, 1, 0.25 pg/ml) was tested. Washout dilutions of 1:10 were tested. * = $P < 0.05$, by Mann-Whitney U test.

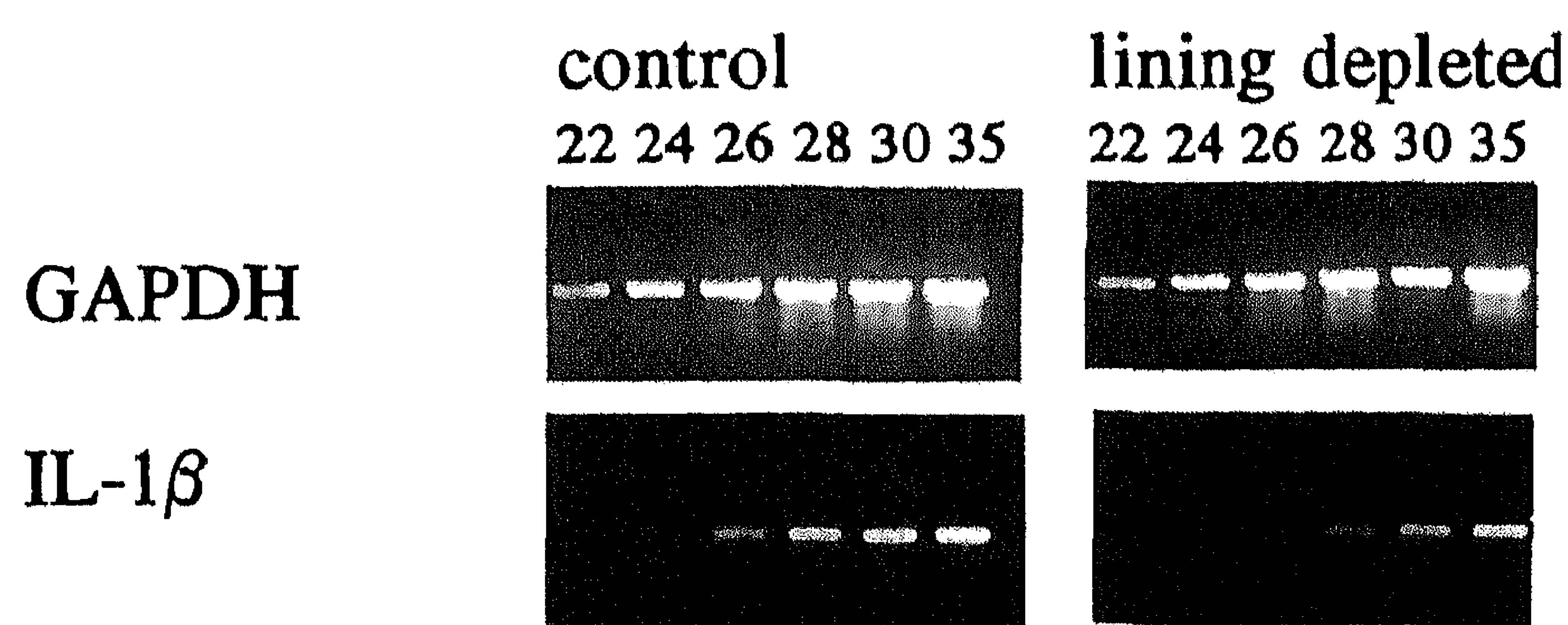


Figure 4. Measurement of interleukin-1 β (IL-1 β) messenger RNA (mRNA) levels in synovial specimens derived from lining-depleted and control arthritic knee joints of DBA/1 mice. Samples from 6 mice per group were pooled and total RNA was extracted. Semiquantitative reverse transcriptase-polymerase chain reaction (PCR) was performed using GAPDH ("housekeeping" gene) as a control. PCR product was analyzed after 22, 24, 26, 28, 30, and 35 cycles. IL-1 β mRNA levels in lining-depleted arthritic knee joints were at least 4-fold decreased (2 cycles).

Figure 7A). In contrast, in the contralateral, lining-depleted knee joints, cell influx was completely absent (Table 3 and Figure 7B).

DISCUSSION

In the present study, we found that selective depletion of phagocytic lining cells prior to arthritis development in CIA causes a significant reduction in infiltration of inflammatory cells into the synovial layer. CIA is a systemic disease in which the arthritis incidence may vary greatly between animals and between the joints within one animal. In general, arthritis expression is higher in peripheral joints than in knee joints. Moreover,

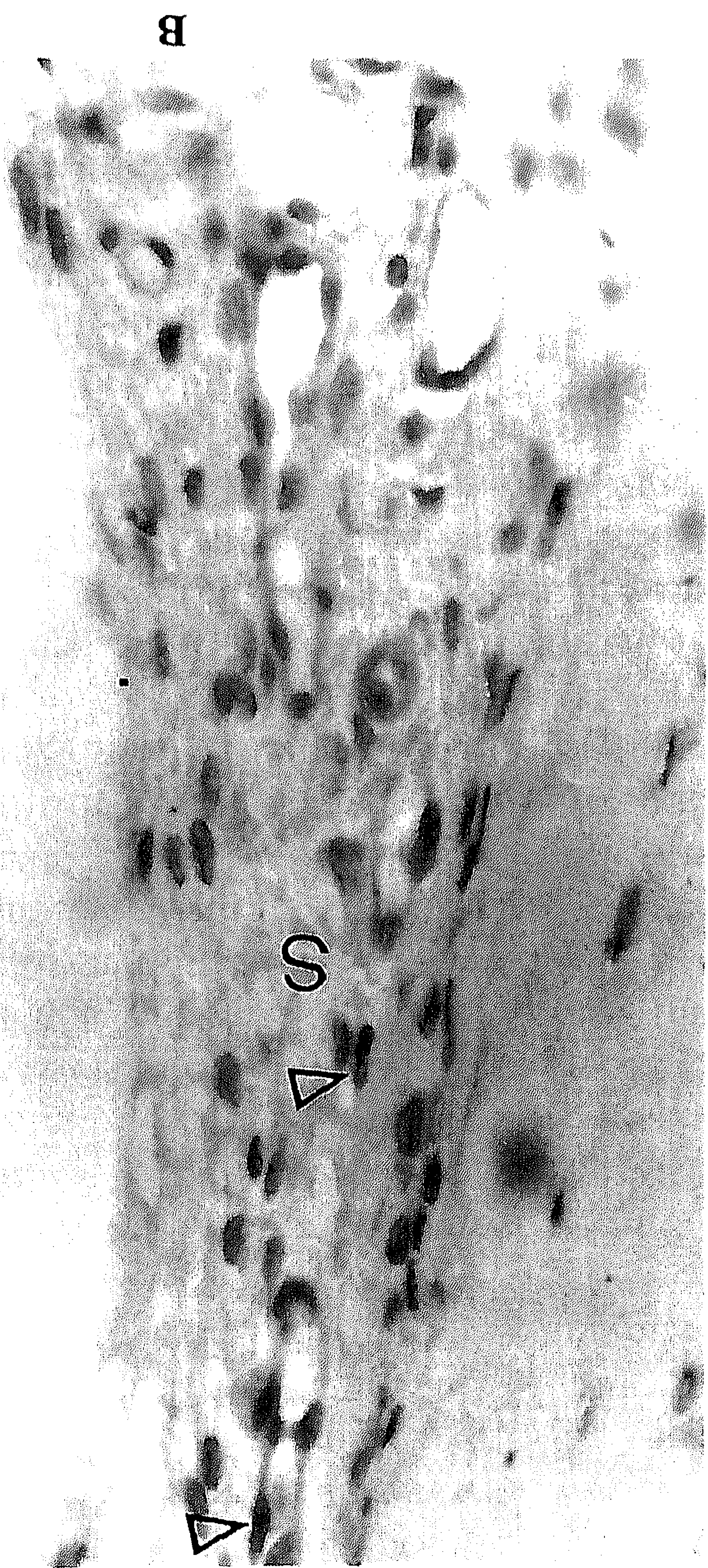
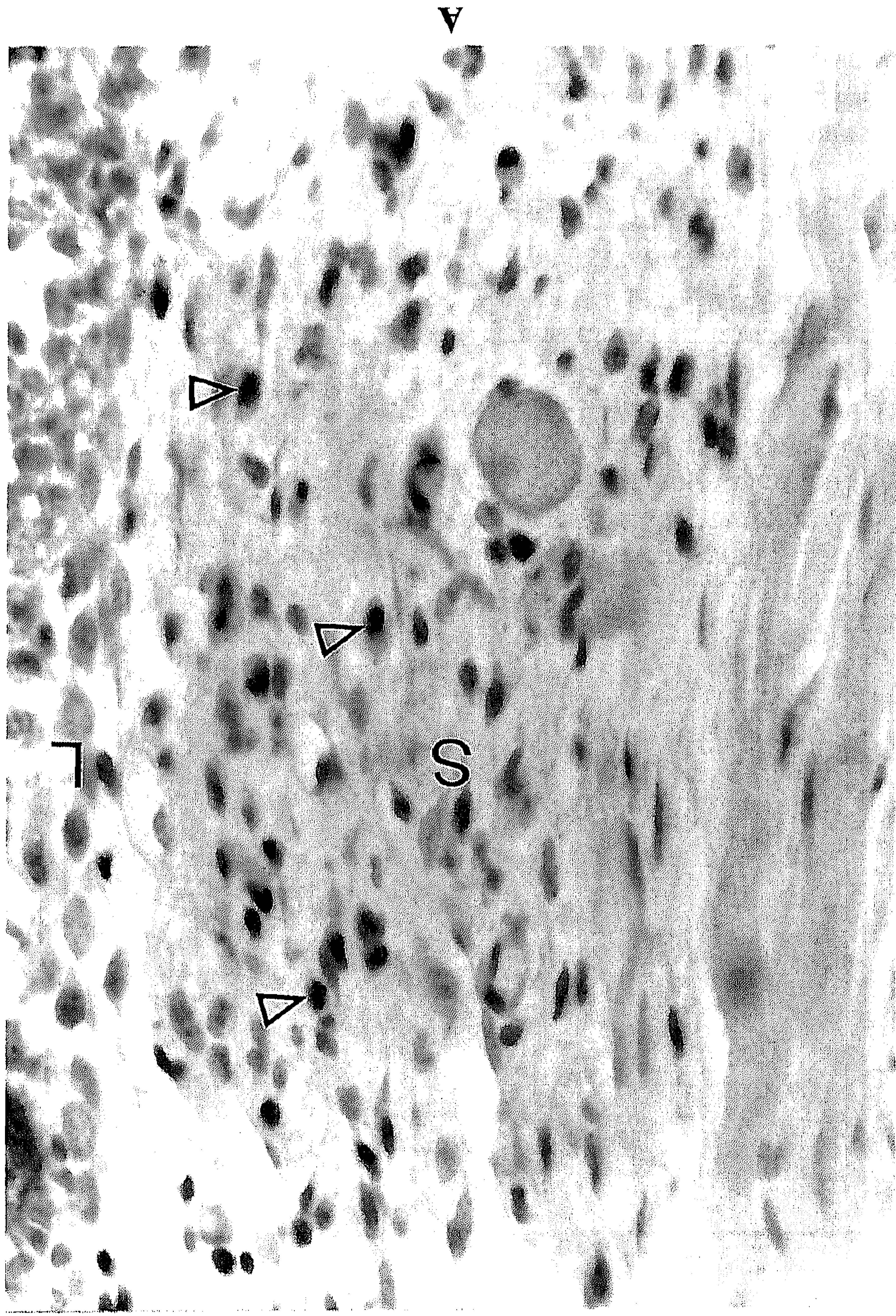


Figure 6. Whole knee joint sections, showing positive staining for interleukin-1 β (IL-1 β) (open arrowheads). Diaminobenzidine (DAB) was used to detect peroxidase-labeled antibodies; hematoxylin and eosin (diluted 1:5) was used for background staining. DAB precipitate is seen as deep black; hematoxylin as grey. Six hours after the onset of arthritis, only cells in the sublining layer of controls stained for IL-1 β (A), whereas positive staining was significantly decreased in lining-depleted knee joints (B). Serial sections stained with normal rat serum or with anti-IL-1 β antiserum preincubated on IL-1 β -coated microtiter plates were negative (data not shown). S = sublining layer; L = lining layer. Original magnification $\times 250$.

arthritis onset is gradual, ranging from day 28 to day 50 after induction. Since a single liposome treatment only transiently eliminates lining cells (13), the lining cells might return before the onset of arthritis in some animals. To avoid this complication, we synchronized the onset of arthritis by administering a single systemic injection of LPS, which accelerates and aggravates arthritis expression (14). Systemically elevated cytokine levels (IL-1 and TNF α) may be responsible for this effect. Both IL-1 and TNF α have been shown to accelerate arthritis (5,8,24). Clodronate-containing liposomes injected into the mouse knee joint have been shown to selectively deplete phagocytic lining cells (13). Macrophages incubated with clodronate-laden liposomes probably die of apoptosis (van Rooijen N, Sanders A: unpublished

observations). Local death of phagocytic lining cells within the knee joint of type II collagen-immunized mice with spontaneous-onset CIA may release proinflammatory cytokines, causing an accelerated onset of arthritis. Interestingly, however, local treatment with clodronate-laden liposomes did not cause arthritis in those knee joints. This suggests that either the release of death by apoptosis limits the cytokine load within the knee joint by keeping cytokines within apoptotic bodies. Lining depletion prior to arthritis induction prevented cell influx for the greater part in the present study. Comparable results were found in immune-complex-mediated arthritis (25,26), which is a locally induced arthritis (27). Reduced cell influx in liposome-treated joints was found both at early (6 days) and at

Table 3. Effects of lining depletion on the onset of type II collagen-induced arthritis after intraarticular injection of IL-1 β or TNF α into the knee joints of DBA/1 mice*

Cytokine injected, joint	Synovitis, mean \pm SD cells
TNF α	
100 ng	
Control	2.1 \pm 1.2
Lining depleted	0.1 \pm 0.2†
200 ng	
Control	2.5 \pm 0.8
Lining depleted	0.4 \pm 0.3†
IL-1 β	
100 ng	
Control	2.5 \pm 0.8
Lining depleted	0.1 \pm 0.2†
200 ng	
Control	2.4 \pm 1.2
Lining depleted	0.1 \pm 0.2†

* Cell influx into synovium was determined 2 days after intraarticular injection of interleukin-1 β (IL-1 β) or tumor necrosis factor α (TNF α) in control and lining-depleted knee joints of type II collagen-immunized mice (day 28; only mice without macroscopic inflammation at that time). Cell influx was scored on a 0–3 scale (0 = no influx, 1 = minor, 2 = marked, 3 = maximal). Cell influx in control knee joints was marked after injection with IL-1 β or with TNF α . In lining-depleted knee joints, cell influx was absent after cytokine injection. Values represent 10 mice per group.

† $P < 0.05$ versus controls, by Mann-Whitney U test.

later (12 days) time points after arthritis onset, indicating a prolonged effect of clodronate-laden liposome treatment. One reason for this might be that it takes some time before the lining cells return to the joint. Earlier studies revealed that even 30 days after a single injection of clodronate-laden liposomes into a normal knee joint, the lining cells in the synovial layer did not return completely (13). However, under inflammatory conditions, the return of lining cells might be somewhat accelerated (28).

Several mechanisms might explain the reduced cell influx in lining-depleted arthritic knee joints. One explanation may be that clodronate-laden liposomes, apart from eliminating phagocytic cells, may also damage endothelial cells, thus influencing cell influx. Earlier studies revealed, however, that clodronate-laden liposomes probably do not reach synovial blood vessels. It was found that fluorescent liposomes did not penetrate the sublining layer, but merely stuck to the lining layer (29). Moreover, injection of recombinant C5a or zymosan into lining-depleted or control joints showed comparable cell influx (26), indicating that cell influx initiated by direct chemotaxis is still possible in lining-depleted knee joints.

Another mechanism may be that in lining-

depleted knee joints, the expression of proinflammatory cytokines is decreased. Both TNF α and IL-1 have been shown to be crucial to the onset of CIA (6,7,9). Both cytokines increase the numbers of adhesion molecules on endothelium and the affinity of integrins on leukocytes for their endothelial ligands (30). Earlier studies done by us (6) and others (7) indicated that IL-1 is the dominant cytokine involved in the expression of CIA. Blockade of IL-1 by anti-IL-1 antibodies or IL-1 receptor antagonist fully blocked the onset of arthritis, whereas blockade of TNF caused only partial relief of inflammation (31).

In the present study, we found that lining-depleted arthritic knee joints showed diminished IL-1 mRNA and protein levels. IL-1 bioactivity was decreased by 40%, whereas only a few IL-1-positive cells were present. The significant decrease in IL-1 production might partly explain the reduced cell influx. However, in contrast to IL-1 injections into control joints, injection of high concentrations of IL-1 into lining-depleted knee joints failed to induce cell influx. Similar results were found using TNF α . Since TNF α is able to induce the expression of CIA, but neutralization of TNF α by anti-TNF α antibodies had only marginal effects on spontaneous arthritis, this suggests that TNF α levels might be too low in the active model. This is consistent with our finding that in arthritic synovial washout samples, no TNF α could be detected. Local injection of neither IL-1 nor TNF caused cellular infiltration in synovial lining-depleted joints. This suggests that in this phase of the arthritis, sublining cells, such as fibroblasts and endothelial cells, are unable to generate sufficient chemotactic factors after activation by IL-1 or TNF. In the prearthritic phase, sublining cells may be less activated than superficial lining cells. This may lead to a gradual decrease of cell surface IL-1 and TNF receptor expression in the deeper synovial layer. Injection of IL-1 or TNF may thus favor lining cells for the production of factors involved in cell influx.

The above results suggest that phagocytic lining cells may be directly involved in cell influx by generating chemotactic factors within the knee joint. Indeed, samples of synovial washouts of lining-depleted knee joints showed a significantly decreased chemotactic activity for PMN at 6 and 48 hours after arthritis onset. Phagocytic lining cells may be important producers of chemokines. Recently, it was demonstrated that chemokines attract subsets of leukocytes in a relatively specific manner. C-X-C chemokines, such as IL-8 or macrophage inflammatory protein 2 (MIP-2), mainly attract neutrophils, whereas C-C chemokines, such as MIP-1 α , mainly attract monocytes. The majority of these chemokines are

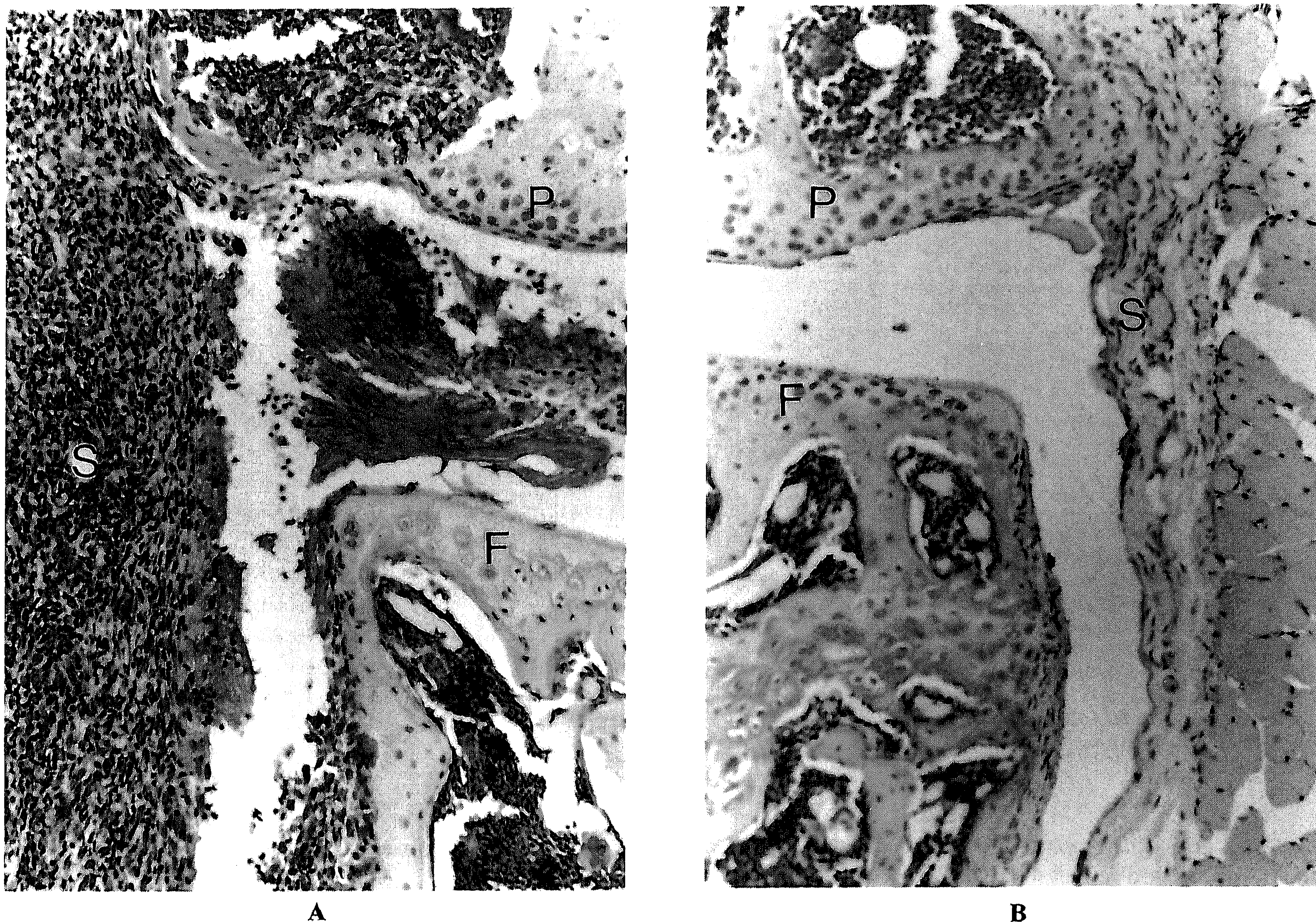


Figure 7. Total knee joint sections of control (A) and lining-depleted (B) arthritic knee joints of DBA/1 mice, 2 days after injection of 100 ng of interleukin-1. Note the absence of cell influx in the lining-depleted knee joint. P = patella; F = femur; S = synovium. Original magnification $\times 100$.

produced by monocytes and macrophages. Anti-IL-8 treatment was shown to reduce cell influx in immune complex-mediated lung inflammation (32), whereas anti-MIP-1 α partially reduced accumulation of mononuclear phagocytes in interstitial lung inflammation (33). In CIA, both classes of chemokines may be important in cell influx. One study showed that lower levels of MIP-1 α and MIP-2 were correlated to a decrease in cell influx in CIA as a result of IL-10 treatment (34). In our study, 75% of the infiltrated cells appeared to be monocytes and 25% PMN, when studied 6 days after the onset of arthritis. In lining-depleted joints, we found that cell influx was decreased by 70%, but the same ratio of monocytes:PMN was found, which indicates that both classes of chemokines might be lowered as a result of lining depletion.

Apart from a diminished influx of monocytes and PMN, T cell influx may also be impaired. Some chemo-

kines produced by lining cells, such as monocyte chemoattractant protein 1 (MCP-1) and RANTES, are chemoattractants for T cells (35,36). The latter cells are involved in the onset of CIA, since blockade of CD4 cells by anti-CD4 antibodies largely prevented the onset of the disease (37). Furthermore, phagocytic lining cells may be involved in antigen presentation to the T cell, thus accelerating the disease.

In summary, this study indicates that phagocytic lining cells seem to be important in directing cell influx. Since macrophages produce both proinflammatory as well as antiinflammatory cytokines (e.g., IL-10), depending on their activation stage, the complete removal of these cells might be too extreme a solution. However, more sophisticated treatment of these cells by drugs encapsulated in liposomes, which selectively block proinflammatory cytokines/chemokines and/or tissue-destroying molecules, might well be a promising tool to

combat the propagation of inflammation during rheumatoid arthritis.

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